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HERMAN BEERMAN LECTURE

THE PARTICIPATION OF CELLS IN THE INFLAMMATORY INJURY OF TISSUE

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Knowledge of the inflammatory process has increased immeasurably in recent years. The broad bases of information now being developed will be of significant help in many fields of medicine, not the least being that of dermatology where inflammatory conditions comprise a significant portion of the total disease spectrum. In this presentation I would like to review some of the more recent developments occurring in various areas of the inflammatory process, underscoring the important concept that the inflammatory process involves a series of sequential interactions between plasma components and cells that lead eventually to the disruption of tissue. The discussion will therefore center around mediation systems of the host as opposed to inciting agents.

Evidence for the Participation of Mediation Pathways in the Development of Inflammatory Injury

It is important first to demonstrate conclusively that an interaction between plasma components and cells indeed exists that is essential to the full development of inflammation resulting from the deposition of an inciting agent. For purposes of illustration, I have chosen immunologic reactants as inciting agents. Abundant evidence now indicates that immune complexes induce the development of glomerulonephritis, Arthus reactions, arteritis, and acute synovitis.

A multistep pathway leading to the injury of tissues has been demonstrated in each of these immunologic reactions. The mediation by these pathways has been found essential to development of injury. Removal of polymorphonuclear leukocytes prevented development of each reaction, and depletion of complement components was found to slow or prevent accumulation of neutrophils resulting in inhibition of the reaction [1]. We will deal with the latter phenomenon in more detail.

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Mechanisms by Which Leukocytes Accumulate in Tissues in Response to Immunologic Stimuli

Studies into the means by which neutrophils accumulate in tissues following formation of immunologic complexes have indicated that the complement system plays an important role. As noted above, depletion of critical complement components prevented accumulation of neutrophils at sites of antigen-antibody deposition [1]. Two general mechanisms by which the complement system exerts this effect have been studied: immune adherence of the leukocytes to the activated, bound third component, C3b, and to probably a lesser extent the bound fourth component and chemotaxis to solubilized complement moieties. In immune adherence, neutrophils as well as monocytes and platelets adhere to bound C3b which may be activated by either classical or alternate pathways of the complement sequence. C3b possesses sites in its structure, as yet uncharacterized, that allow firm binding with receptors on the above-mentioned cells. Specificity of the interaction is demonstrated by the fact that other cells, e.g., T lymphocytes, do not bind to the fixed C3b.

Chemotaxis is the second complement-dependent mechanism of neutrophil accumulation. Other cells, e.g., monocytes and perhaps basophils, also respond to activated complement components. The fragments C3a, C5a, and $\overline{C567}$ act to stimulate cells chemotactically. Again a distinct specificity is observed. Some leukocytes such as lymphocytes fail to respond to these chemotactic agents. Chemotactic activity may also be demonstrated with an ever-enlarging number of agents: kallikrein, plasminogen activator, a bacterial chemotactic factor derived from several different species of organisms which is released into the supernatant fluid of the growing culture of the bacteria, from casein, etc.

But while in the past years there has been a large number of biologically important chemotactic factors demonstrated in vitro, there has been a dearth or complete absence of information on whether chemotaxis takes place in vivo that could explain the development of immunologic lesions. Toward this end, a model of experimental synovitis in rabbits was developed [2]. Rabbits were depleted of polymorphonuclear leukocytes with nitrogen

mustard, and antigen-antibody reactions were then induced in small blood vessels up to 200 μ or greater in distance from the synovial space in the surrounding tissues. Neutrophils were then carefully prepared from the peripheral circulation of other rabbits and were infused into the joint space of the rabbits bearing antigen-antibody deposits in the synovial tissue blood vessels. It was observed that the neutrophils were capable of migrating from the joint space through the synovial tissues to find antigen-antibody complement deposits up to 200 μ away from the joint space. Rabbits were then used that were either depleted of the third component of complement by use of the cobra venom factor or were genetically deficient in the sixth component of complement. It was found that the infused neutrophils did not migrate to the site of antigen-antibody interaction at nearly the rate they did in normal rabbits (Fig. 1). If the C6-deficient rabbits were depleted of C3 with cobra venom factor, migration then was completely blocked. This indicated that the migration of neutrophils through the tissues of the synovial membranes to a deposit of antigen and antibody requires the presence of the full complement system for their chemotactic migration [2]. This presumably constituted a reasonable representation in vivo for what had been previously demonstrated in chemotactic chambers in vitro.

Thus two mediators, complement components and neutrophils, were found essential for development of these lesions. The infiltration of neutrophils into areas of myocardial ischemia in rats is also inhibited when the animals are depleted of complement with cobra venom factor. This indicates complement is important in lesions caused by inciting agents other than immunologic reactants.

The Participation of Inflammatory Cells in Tissue Injury

Much evidence now indicates that neutrophils exert their effect by releasing injurious constituents at the site of injury. Among the injurious constituents can be numbered proteases, collagenase, elastase, cationic proteins, hydrogen ions, etc. [3]. These constituents have been shown to increase vascular permeability, degrade membranes of blood-vessel walls, cleave anaphylatoxins and chemotatic factors from their parent proteins of complement system, activate mast cells to release histamine, etc. [3]. It is of interest to examine the mechanisms by which these and other inflammatory cells release their constituents and by which they are stimulated to effect the release.

The release of injurious constituents from inflammatory cells. When leukocytes are brought into contact with the antigen-antibody aggregate they are stimulated to release a wide variety of constituents. Perhaps the largest number of these constituents is actually preformed and packaged in membrane-bound granules, some of which are properly called lysosomes in the case of monocytes, neutrophils, and platelets. The release of injurious constituents that occurs is, in almost every case, by a process of exocytosis in which the membrane surrounding the granule fuses with the external membrane of the cell. An opening then develops to the outside, allowing the injurious granular constituents to escape into the surrounding medium. The cell thereby survives the process. This extrusion of granules is demonstrated in Figures 2 and 3.

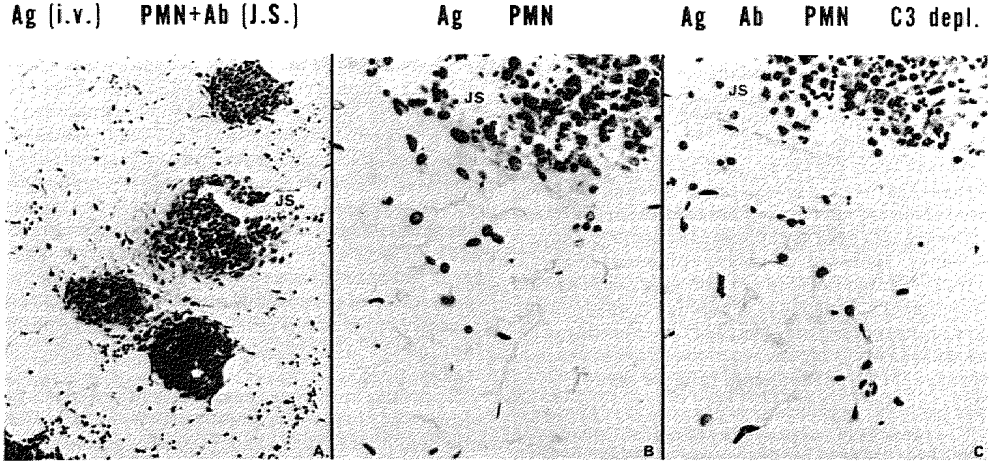

FIG. 1. Evidence of chemotactic movement of neutrophils in vivo. (*A*): antigen-antibody-complement contraction (Arthus reactions) took place in small blood vessels in the synovial tissues of a neutrophil-depleted rabbit. Purified neutrophils were then infused into the joint space (J.S.) and migrated to the affected blood vessels where they became bound as noted by the dense accumulation of these cells in the figures. In (*B*), antigen was omitted and the migration and accumulation did not take place. In (*C*) the complement (C3) was depleted with cobra venom factor in a rabbit treated the same as that in (*A*). The neutrophils in the joint space were not attracted to the blood vessels where the antigen-antibody reaction took place. (From [2].)

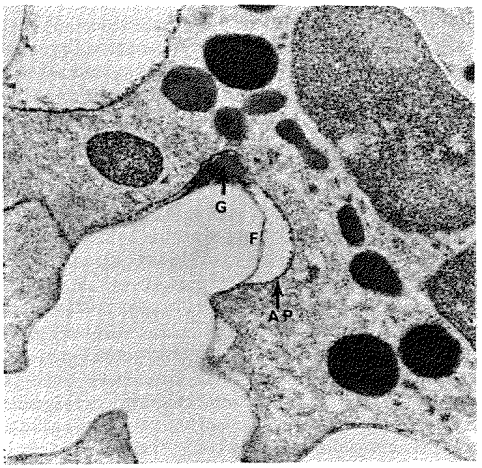

FIG. 2. Electron photomicrograph of a neutrophil adhering to the convoluted surface of a micropore filter (*F*) to which aggregated immunoglobulins are adherent. The neutrophilic granules (*G*) are being released along the surface where stimulation is occurring. Note fusion of the granular membrane and the external membrane of the cell. The black deposit lying along the neutrophilic membrane is alkaline phosphatase (*AP*) which derives from granules already discharged to the surface. (From [8].) An identical picture is seen when neutrophils adhere to the glomerular basement membrane in acute immunologic glomerulonephritis [1].

A multistep mechanism takes part in the release of injurious constituents from cells. These will be dealt with separately:

1. *Proesterase enzymes.* In each of the systems mentioned above it has been found that when the activator is added to the cells in the presence of diisopropylfluorophosphate (DFP), the release of the constituents of the cell does not occur [4]. As a control, when the cells were pretreated with diisopropylfluorophosphate and then washed prior to the addition of activators, the cells were still fully susceptible to the action of the activator and release occurred. Dr. E. L. Becker and his colleagues have also studied a group of organophosphorous (phosphonate) inhibitors having varied capacity to inhibit esterases. A class of inhibitors bearing side chains of varying length makes it possible to differentiate between esterase enzymes by the distinct patterns of inhibition. This has allowed the differentiation of proesterase enzymes in the cells that undergo exocytosis of granules and release of injurious constituents. In the case of chemotaxis of neutrophils, direct evidence for the importance of esterase enzymes has been obtained. Certain synthetic esters when added to these cells in the presence of the chemotactic activator are cleaved in the reaction process. The cleavage of the synthetic ester has been related to the activation. Evidence for a DFP-inhibitable activation of esterase has been obtained with platelets and mast cells (reviewed in [4]). The data indicate that the proesterase-esterase conversion is an early event in the release of granules from certain of these cells [5].

2. *Energy systems.* For the release of injurious constituents by each of the cell types noted, an active energy source is essential [4]. Whether oxidative phosphorylation or anaerobic glycolysis or both are employed by the cell varies from cell type to cell type. Mast cells apparently require both anaerobic glycolysis and oxidative phosphorylation, for the blockade of either one alone fails to inhibit the cell from its release. A similar situation is obtained with platelets. However, in the case of neutrophils, the various functions of the cell are governed almost entirely by anaerobic glycolysis.

3. *The presence of divalent cations.* In each of the release systems of the various cells noted, calcium is required in the external medium. In most systems where the tests have been carried out, magnesium is required as well.

4. *Intracellular cyclic AMP and GMP.* In their pioneering work on the release mechanisms from basophils in peripheral human leukocytes, Lichtenstein and Margolis found that beta adrenergic stimulating agents and antagonists of intracellular

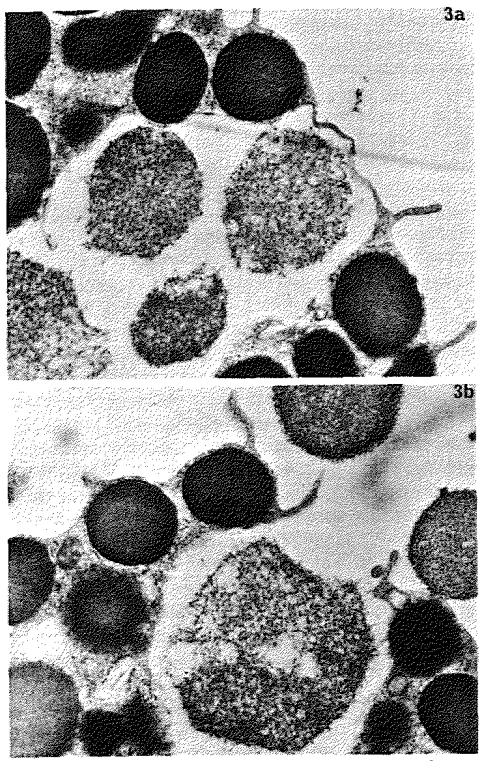

FIG. 3. Electron photomicrographs of rat peritoneal mast cells exposed to human C3a (anaphylatoxin). (*a*): The histamine-containing granule is in the process of exocytosis. The membrane surrounding the granule will fuse with the external membrane of the cell. Rupture of the external membrane at that point occurs and the granule is discharged as shown in (*b*). Loss of cytoplasm is avoided and the cell survives.

phosphodiesterase inhibited the release of histamine from the leukocytes [6]. The effect of both together was synergistic in inhibition, and was related by the authors to levels of intracellular cAMP: when cAMP levels were elevated, release of histamine was inhibited. This finding was in contradistinction to the classical work of Sutherland (see [7]) on hormonal systems in which the same agents augmented the release of hormones from the cells upon stimulation. The studies of Sutherland related the stimulation of release to an increase in the intracellular levels of cyclic AMP. The work of Lichtenstein has been extended to neutrophils, platelets, pulmonary and peritoneal mast cells, and macrophages in numerous other laboratories (see review [4]). In general, these have shown that the levels of cAMP exert a dynamic control over the release of their lysosomal constituents. While elevated cAMP levels have inhibited release of granules, elevation of cyclic GMP through cholinergic stimulation has been found to augment release. Thus, a double controlling mechanism is exerted by the cyclic nucleotides within the cells. The importance of these mechanisms in therapeutic intervention is apparent.

5. *Microtubules and microfilaments.* Intact microtubules have been shown essential for maximal release of granular constituents of inflammatory cells. Colchicine and vinblastine, for example, which lead to disaggregation of microtubules, inhibit release (see [4]). By the same token, in some of the studies the use of deuterium oxide which stabilizes the microtubule actually enhances the release of injurious constituents. In the case of microfilaments, the use of cytochalasin B as a disaggregator has been almost exclusively used as an inhibitor. In general, an increased release of cellular constituents occurs. The action of cytochalasin B involves other cellular systems as well, and therefore its effects are nonspecific. Thus, the role of microfilaments in the release process must await further experimentation.

Mechanisms of stimulation of inflammatory cells to release injurious constituents. The process by which immunologic activators or humoral effector molecules stimulate inflammatory cells to migrate directionally in chemotaxis, to bind to bound C3b in immune adherence, and to release injurious products is a subject of current investigation. From small peptides or polyamines to macromolecular activators, a large spectrum of soluble agents exists that exhibits the capacity of activating these cells. There are many questions which arise. Do the activators or stimuli induce the changes, i.e., chemotactic movement, phagocytosis or exocytosis of granules, by surface contact, or is internalization of the stimulus required? Do actual receptors exist on the membrane for the activators or is there nonspecific uptake and subsequent recognition of the stimulus? If receptors exist, are they specific for each activator or is there a spectrum of activators recognized by a few receptors on the cell? Finally, what is the nature of the receptors if they exist, what is their chemical composition, by what mechanism do they bind an activator, and what is the nature of the change in the receptor site that then sets in motion events within the cellular cytoplasm? Most of these questions are unanswered. However, data are beginning to accumulate as will be dealt with in the remainder of this discussion.

Does activation take place at the cell surface, or is internalization of the stimulus required? Information is now available indicating that surface contact with the activator is sufficient to stimulate fully certain functions of the cells. Platelets, after contacting insoluble collagen, bind to the collagen and release nucleotides and vasoactive amines. The same effects follow contact between zymosan particles coated with the activated third component of complement (C3b), and rabbit platelets. Neutrophils that are brought down onto a membrane coated with tightly bound aggregated γ globulin or ferritin–antiferritin complexes, discharge their cytoplasmic granules containing proteolytic enzymes to the outside of the cell as mentioned above (Fig. 2) [8]. Electron microscopic observations confirm that the ferritin–antiferritin complexes stimulate without being taken into the cell. Thus, with two types of cells that participate in the inflammatory process, contact between the activator and the surface of the cell leads to exocytosis of granules and release of biologically active constituents. In vivo, the analogy between neutrophils and surface activation is readily observed with neutrophils that adhere to immunoglobulins and C3b along the glomerular basement membrane. Exocytosis of granules occurs with release of proteolytic enzymes and severe injury of the basement membrane.

Considerable specificity of certain classes of immunoglobulins exists in their capacity to stimulate release of constituents of various inflammatory cells. Human immunoglobulins of the $IgG_{1,2,3,4}$ and IgA, upon aggregation, induce the exocytosis of granules from neutrophils. IgM, IgD, and IgE are inactive [9]. In platelets, $IgG_{1,2,3,4}$ but not the other immunoglobulins stimulate. The exocytosis of granules is also readily observed in the case of monocytes that enter tissues in response to an inflammatory stimulus. In the case of mast cells and basophils, IgE immunoglobulins (and in the rat, IgG_2), but not other immunoglobulins, stimulate release of constituents. Thus, one observes specificity of stimulation by immunologic reactants of the inflammatory cells that are essential to various injurious processes.

An extremely effective augmentation of the stimulation for release of constituents of neutrophils was observed by my colleague Dr. Peter Henson [8] when the immunoglobulins, in aggregated form, were adsorbed to a nonphagocytosable surface prior to exposure to the cells. Such stimulation is over 20 times more active than when

neutrophils contact the aggregates in suspension [8]. In addition, the smallest aggregates, which fail to stimulate release of neutrophilic contents in suspension, are potent stimulators when adsorbed to a surface. The meaning of this is underscored when it is realized that neutrophils undoubtedly contact aggregated immunoglobulins on surfaces in vivo, for example along the glomerular basement membrane or in interstitial spaces.

In the case of mast cells, recent evidence accumulated by Dr. David Morrison in this laboratory indicates that low-molecular-weight activators stimulate the cells on the surface of the membrane to release their granules and, thereby, histamine and serotonin [10]. Polymyxin B, when covalently coupled to Sepharose 4B beads and exposed to rat peritoneal mast cells, induced binding of the mast cells to the surface of the Sepharose beads and exocytosis of the mast cell granules. Chemical assays indicated that undetectable quantities of polymyxin B were released into the medium from the insoluble beads. The sensitivity of the chemical assay indicated that less than $\frac{1}{20}$ of the amount of polymyxin could have been released that was necessary for threshold stimulation of the mast cells.

As to the question of whether distinct receptors for soluble activators exist on leukocytes, Dr. Morrison found that radiolabeled 48/80 became bound to rat peritoneal mast cells (but not macrophages), and that nonradiolabeled 48/80 or polymyxin B were able to block the uptake of the radiolabeled 48/80 [11]. By contrast, C3a anaphylatoxin, another activator of mast cells, and a protein isolated from cobra venom were unable to block binding of the 48/80. The activators could therefore be divided into two types, 48/80 and polymyxin B are type I activators, and C3a and cobra venom protein are type II. It was also observed that type I activators would render the mast cells insensitive to other type I activators, but that the cells were still susceptible to activation by type II activators; and conversely, treatment with type II activators rendered the mast cells insensitive to other type II agents, but left them susceptible to type I activators (Tab.). These studies indicate that there are two distinct receptors on rat peritoneal mast cells which bind activators. It should now be possible to study the binding sites, to determine binding affinities, and eventually to characterize the binding site. Hopefully, in such studies the molecular counterparts of the initiation of activation will come into focus. The therapeutic importance of such knowledge cannot be overstated. Chemical analogues of the activators might well be synthesized that bind to the receptor site, fail to activate the cell, and prevent interaction with potentially active stimuli. That such analogues exist is exemplified by C3a which loses its capacity to activate mast cells when the C-terminal arginine is cleaved, but still binds to the cell.

As noted above, serine esterase enzymes that are

TABLE. *Desensitization of mast cells by type I and type II activators*

Initial incubation (% release)	Second incubation (% release)		
	Compound 48/80	CVA protein	Buffer
Compound 48/80 48.9	6.7	20.4	5.1
CVA protein 27.0	47.7	5.0	5.2
Buffer 2.1	61.3	29.5	2.1

essential for chemotaxis, phagocytosis, and exocytosis of granules are stimulated during the activation process. These serine esterases act early in the process of exocytosis of granules from mast cells [5]. Their relationship to the mechanism of activation is not clear. However, recent studies by Henson et al [12] have indicated that five different stimuli of platelets activate different serine esterases as determined by the inhibition spectra obtained with Becker's phosphonate inhibitors and with inhibition by trypsin inhibitors of plant origin and by synthetic substituted esters (benzoyl larginine ethyl ester, etc.) acting competitively to evoke inhibition. In preliminary studies with rat peritoneal mast cells using two forms of anaphylatoxins (human C3a and rat anaphylatoxin), and band 2 protein of rabbit neutrophils, a different pattern of esterase inhibition was observed by the author with one class of phosphonate inhibitors.

Thus evidence of a distinct specificity is emerging of not only the receptor site on the surface of inflammatory cells, but also the serine esterase enzymes that are activated shortly after stimulation occurs. The relationship of the receptor site to the serine esterase is a question of great significance and intense inquiry.

SUMMARY AND CONCLUSION

Numerous endogenous and exogenous agents have been identified that stimulate inflammatory cells to migrate, phagocytize particles, and discharge their granules and injurious constituents to the outside. This process constitutes one of the fundamental and important facets of inflammation. When the stimuli are associated with a surface, the release of injurious constituents from the inflammatory cells is greatly heightened.

The processes of exocytosis of cytoplasmic granules, phagocytosis, and chemotaxis, require serine esterase enzymes, ATP generating systems, and divalent cations, and in general appear to be facilitated by an intact microtubular system, low levels of intracellular cAMP, and elevated levels of cGMP.

At the forefront of research activity in inflammation is the analysis of the mechanism of activation of cells by humoral stimuli. Evidence is accumulating to indicate that distinct receptor sites exist on the surface of inflammatory cells that bind specific humoral activators. Stimulation of the cell results from activation at the surface of the

cell. Evidence has been presented indicating that immunoglobulin, firmly bound to a surface, causes neutrophils to extrude their granules along the surface of the cell where stimulation occurs. Mast cells respond to polymyxin B bound firmly to Sepharose beads in a similar fashion, and platelets extrude their dense granules when contacting activators adherent to particulate receptors. Specificity of recognition sites on the membranes of neutrophils, platelets, and mast cells is apparent. In the latter situation, the specific binding of low-molecular-weight activators is described.

REFERENCES

1. Cochrane CG: Immunologic tissue injury mediated by neutrophilic leukocytes. Advances in Immunology. Edited by HG Kunkel, FJ Dixon. New York, Academic, 1968, pp 97–156
2. DeShazo CV, McGrade MT, Henson PM, Cochrane CG: The effect of complement depletion on neutrophil migration in acute immunologic arthritis. J Immunol 108:1414–1419, 1972
3. Cochrane CG, Janoff A: The Arthus reaction: a model of neutrophil and complement mediated injury, The Inflammatory Process. Second edition. Edited by BW Zweifach, L Grant, RT McCluskey. New York, Academic, 1974, pp 85–102
4. Becker EL, Henson PM: *In vitro* studies of immunologically induced secretion of mediators from cells and related phenomena, Advances in Immunology. Edited by HG Kunkel, FJ Dixon. New York, Academic, 1973, pp 93–193
5. Ranadive NS, Cochrane CG: Mechanism of histamine release from mast cells by cationic protein (band 2) from neutrophil lysosomes. J Immunol 106:506–516, 1971
6. Lichtenstein LM, Margolis S: Histamine release *in vitro*: inhibition by catecholamines and methylanthrines. Science 161:902, 1968
7. Sutherland EW, Robison GA, Butcher RW: Some aspects of the biological role of adenosine 3′,5′monophosphate (cyclic AMP). Circulation 37:279–292, 1968
8. Henson PM: Interaction of cells with immune complexes: adherence release of constituents, and tissue injury. J Exp Med 134:114s–135s, 1971
9. Henson PM, Johnson HB, Spiegelberg HL: The release of granule enzymes from human neutrophils stimulated by aggregated immunoglobulins of different classes and subclasses. J Immunol 109:1182–1192, 1972
10. Morrison DC, Roser JF, Cochrane CG, Henson PM: The initiation of mast cell degranulation by activation at the cell membrane. J Immunol 114: (in press) 1975
11. Morrison DC, Roser JF, Cochrane CG, Henson PM: Evidence of two modes of initiation of mast cell degranulation. J Immunol 114: (in press) 1975
12. Henson PM, Gould D, Becker EL: Activation of stimulus-specific serine esterases in the release reaction of rabbit platelets (abstr). Fed Proc 33:798, 1974